93. The Estimation of Protein by the Biuret and Greenberg Methods

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For the estimation of small amounts of protein in biological fluids such as serum, urine and cerebrospinal fluid, as well as in certain foods such as milk, there have been many attempts to utilize colour tests. Perhaps the two most important of these are the biuret test, and the test for tyrosine introduced by Folin and his colleagues, and applied to serum, albumin and globulin by Greenberg [1929], who matched the colour against that produced by a standard tyrosine solution, factors being used to correct for the different tyrosine content of the two proteins. Fine [1935] applied the biuret test to these two serum proteins. The colour produced was matched in a colorimeter against that produced by a standard serum of which the protein content had been determined by Kjeldahl estimations. No correction factor was employed to distinguish between albumin and globulin, since equal weights of these were stated to produce equal intensities of colour. Lieben & Jesserer [1936] used the Zeiss-Pulfrich step-photometer to investigate the biuret test, and found that equal weights of casein, gelatin, egg white, fibrin, edestin and zein reacted with the same amount of copper. The biuret colour was said to be affected by the *p*H and by the concentration of Cu ions in the reaction mixture, the best results being given when a 3% NaOH and a 1% $CuSO_4$ solution reacted with 0·5% of protein. The biuret colour was stated to consist of two components, a red and a blue. The red component was stated to be characteristic of protein and was measured by means of a No. 47 filter, which showed an increase in this red component as the protein solution aged, but no figures were given for this increase. Sizer [1937], using a recording photoelectric spectrophotometer, determined the absorption band of the biuret colour given by casein, and found a maximum at about 520 mμ. Harrison [1937] introduced a simplified biuret test for estimating protein in serum, urine, cerebrospinal fluid and milk. The colour was matched against a graded series of coloured glasses in a Lovibond comparator. An equal weight of each protein was assumed to produce the same intensity of colour. Robinson, Price & Hogben [1937] returned to Fine's method of matching the biuret colour in a colorimeter against that given by a standard serum preserved with thymol, and introduced certain improvements in the technique. Robinson & Hogben [1940] repeated Sizer's spectrophotometric investigation of the biuret colour given by serum and obtained a similar band with a very blunt peak. They considered the maximum to be at 560 mμ, though their published curve showed no significant difference in density between 540 and 560 mμ. The biuret colour on standing 24 hr. changed from purplish red to red, but no difference was observed in the density values at 560 mμ up to 48 hr. Loss of protein occurred on filtration. Variation of the amount of $CuSO_4$ added from 1·25 to 2·5 ml. of a 20% solution per 100 ml. made no difference to the colour, but when 25 ml. of 2% solution was used the results became more variable and were influenced by the concentration of NaOH. The results obtained by their standard technique agreed with protein estimations by Kjeldahl over a wide range of samples.

Experimental

Tests on numerous clinical samples of urine and cerebrospinal fluid having shown that precipitation methods (e.g. Esbach, Aufrecht, Mestrezat) gave unreliable results, we turned our attention to colour tests. Greenberg's method was applied as described by Greenberg [1929]. The biuret test was employed as directed by Harrison [1937] with slight modifications mentioned below, where we also describe the calibration of the Lovibond glasses we used. This calibration showed that one of the chief sources of error in Harrison's method may be the inaccurate grading of Lovibond glasses, and special precautions were necessary to minimize the error. The other sources of error in both methods were minimized by adjusting the conditions until statistical analysis of the results showed that the percentage standard deviation had been made as small as possible. This required a large number of readings, especially when using the colorimeter. In fact, most of the results we quote are based each on some 50 or 60 readings taken independently by two different observers, thus ensuring an average percentage standard deviation ranging from 0·8 to 1·2 in our Greenberg results, so that a difference of 2 to 3 % (2½ times the standard deviation) was considered significant. The biuret test by Harrison's method is much less accurate, the average percentage standard deviation being at least three times that for the Greenberg method, so that a percentage difference of less than 8 was not significant.

Estimation of total protein in serum

The total protein was estimated by the two colour tests in a number of dilutions of normal horse serum, of which the protein content had been determined by Kjeldahl determinations, using the protein/N factor of 6·55 proposed by Adair & Robinson [1930], after deducting the non-protein N which was determined separately. The results, some of which were obtained on a serum which was a few days old, are given in Table 1. They showed the following points.

1. The biuret method becomes unreliable when applied to dilutions below 1 in 50 (which frequently occur in clinical samples).
2. Omitting results below 1 in 50, the percentage standard deviation of the biuret method was 8·3, within the limits to be expected from statistical analysis of individual readings.
3. The percentage standard deviation for the Greenberg results was also within the expected range.
4. The mean result for the Greenberg estimations was significantly less than 100 % of the true value. If, however, Adair and Robinson's factor of 6·55 was replaced by the usual protein factor of 6·25, the mean for the Greenberg results became 94 % of the true value.
5. The mean result for the biuret method was significantly less than the true value.

Table 1. *Estimation of total protein in serum by biuret and Greenberg methods*

Results given as percentages of the true value as determined by Kjeldahl estimations.

Dilution of serum	Biuret	Greenberg
1 in 10	75	90
1 in 20	81	88
1 in 50	88	91
1 in 100	68	90
Mean	78	90
Standard deviation	6·5	1·4
% deviation	8·3	1·5

Table 2. *Estimation of total protein in different samples of normal serum by biuret and Greenberg methods*

Serum	Approx. age of serum (weeks)	Biuret	Greenberg
Horse II	1	109	78
Horse III	2	81	90
Human I	4	102	95
Human II	1	65	84
Mean		89	87
Standard deviation		20	7·4
% deviation		22·5	8·5

Estimation of total protein in different sera

The total protein of several samples of normal horse and human serum was estimated by the two colour tests. The results, again expressed as percentages of the true value as determined by Kjeldahl determinations, are given in Table 2. It will be seen that the

values obtained on fairly fresh sera were significantly lower than the true values, and that the estimations on older sera gave results which agreed with, or were greater than, the true values.

There was a definite increase in the values given by both methods as the serum aged.

Estimation of albumin and globulin in normal horse serum

The albumin and globulin were separated by Greenberg's sodium sulphate method, and the total protein was estimated in the two fractions by the biuret, Greenberg and Kjeldahl methods. Two separations (1st and 2nd) were made within a day or so of each other, then after an interval of several weeks two further separations (3rd and 4th) were again made within a day or so of each other. The serum throughout all our experiments was stored in a refrigerator and showed no signs of decomposition. The results of these experiments, again expressed as percentages of the true values, are given in Table 3, and from them the following facts emerge.

1. There was a definite increase in the values obtained on both protein fractions by both methods as the serum aged.
2. Results obtained within a day or so of one another agreed within the expected limits.
3. The biuret method gave higher results on albumin than on globulin, in parallel determinations.
4. The mean biuret result was significantly higher than the mean Greenberg result.

Some of the Greenberg results are omitted because the standard used was afterwards found to be unreliable.

Table 3. *Estimation of albumin and globulin in normal horse serum by biuret and Greenberg methods*

Results given as percentage of the true value as determined by Kjeldahl estimations.

	Approx. age of serum (weeks)	Albumin		Globulin	
		Biuret	Greenberg	Biuret	Greenberg
1st separation	4	97·8	79·7	92·2	—
2nd ,,	4	96·4	—	94·3	—
3rd ,,	8	122	88·2	109·2	82·7
4th ,,	8	127	89·1	113·8	84·5
Mean		110·8	85·7	102·4	83·6
Standard deviation		15·9	3·0	10·7	1·3
% deviation		14·3	3·5	10·5	1·5

Table 4. *Estimation of albumin and globulin in normal human serum by biuret and Greenberg methods*

Results given as percentages of the true value as determined by Kjeldahl estimation.

Citrated serum	Approx. age of serum (weeks)	Albumin		Globulin	
		Biuret	Greenberg	Biuret	Greenberg
1st separation	3	102	79·2	52·9	82·6
2nd ,,	6	114	83·2	83·8	88·5
3rd ,,	6–7	105	81·0	70·8	89·7
Mean		107	81·1	69·2	86·9
Standard deviation		6·2	2·0	18·1	3·8
% deviation		5·8	2·5	26·2	4·4
Oxalated serum					
1st separation		90·4	83·4	76·2	90·3

Estimation of albumin and globulin in normal human sera

Similar experiments were carried out on normal human sera. The first sample used was obtained from citrated blood collected for transfusion purposes and stored aseptically in the refrigerator for 3 weeks before we examined it. The first separation of the proteins

in this sample was made when it was 3 weeks old, the second when it was nearly 6 weeks old and the third when it was between 6 and 7 weeks old. The second sample of human serum was obtained from freshly collected oxalated blood, separated within an hour of collection and examined a few days later. The results, given in Table 4, on the whole confirmed those already obtained on horse serum in showing that as the serum aged the results by both methods became higher, and that the biuret method gave higher results on albumin than on globulin.

Precautions taken with the tyrosine standard

The results we had obtained by Greenberg's method might be attributed to our tyrosine standard being (*a*) weak, or (*b*) unstable. Precautions were taken to avoid both of these possibilities. Tests showed that the sample of tyrosine used as standard was of satisfactory purity, and that the solutions of this tyrosine made freshly every week or two and stored in the refrigerator showed no deterioration when checked against new solutions. Moreover, when discussing this point with Dr Rimington, who had also been using tyrosine as a standard in the Greenberg method, we found that he also had obtained low results similar to ours. We therefore concluded that the low results were due to a gradual change in the serum, and tested this conclusion by direct experiment.

Effect of 'age' of serum on Greenberg results

A sample of normal oxalated blood was centrifuged immediately after collection to separate the serum, and the total protein was then estimated in the serum by Greenberg's method. The time from the removal of the blood from the vein to the point when half the readings had been taken on the Klett colorimeter was approximately 60 min., which was considered to be the 'age' of the serum when the first estimation was made. The estimation was repeated at intervals until at the end of 40 days the change in the serum, which was stored in the refrigerator, had practically ceased. The results by the Greenberg method were then 88% of the true values (Table 5). Out of 21 results obtained by the

Table 5. *Effect of age of serum on results given by Greenberg's method for total protein*

Results given as percentages of the true value as determined by Kjeldahl estimations.

Age of serum	Greenberg result	Age of serum	Greenberg result
1 hr.	67	23 hr.	81
2 ,,	74	3 days	83
3 ,,	75	4 ,,	87
4½ ,,	79	40 ,,	88
22 ,,	80		

Note. The 'age' of the serum was calculated from the time when the blood was removed from the vein to the time when half the readings had been taken on the Klett colorimeter, on the assumption that changes might still be taking place in the protein after it was precipitated. It might be more accurate to calculate the age up to the time when the protein was precipitated. This would reduce each of the 'ages' in the above table by about 30 min.

Greenberg method on different samples kept for times ranging from a few days to several months and recorded in Tables 1–4, only one was significantly higher than 88%. Thus it appears that the Greenberg method usually gives results lower than the true value, but it would not be safe to assume that this will always occur, since certain factors such as storage conditions might cause the change in the serum to proceed further. Moreover, if the usual protein/N factor of 6·25 is employed instead of Adair and Robinson's factor of 6·55, the highest results obtainable by Greenberg's method will be at least 5% higher than those recorded above.

Effect of age of serum on biuret results

This effect was more difficult to investigate because of the greater experimental error of the biuret method as employed by Harrison. Statistical analysis of the data from a large number of biuret estimations showed that a difference of less than 6 or 7 % could not be considered significant, and after calibrating the Lovibond glasses we had been using we realized that the experimental error might be much greater. This led us to try the procedure of Fine [1935] and of Robinson & Hogden [1940], of matching the biuret colour in a Klett colorimeter against the colour produced by a standard serum. For this purpose we used a serum several weeks old in which, by analogy with the Greenberg results, the change might be expected to have practically ceased, or at any rate to be progressing so slowly that results obtained within a few hours of each other using it as a standard would be comparable. We found this gave much greater accuracy than the Lovibond glasses, but the experimental error was still more than twice that of the Greenberg method, largely because of the difficulty of matching the blend of two colours. We also introduced the following modifications of Harrison's technique: (1) a 1 in 10 dilution of the serum was employed, of which 2 ml. could be measured more accurately than 0·2 ml. of undiluted serum; (2) after precipitating the protein with trichloroacetic acid and centrifuging, the supernatant fluid was passed through glass wool in a small funnel to collect any particles of protein inadvertently brought over. These particles were washed with distilled water, and the funnel was then placed on the centrifuge tube containing the bulk of the precipitated protein; 1 ml. of 30 % NaOH was dropped on to the funnel to dissolve and carry through any protein particles on the glass wool which was then rinsed with 2 or 3 ml. distilled water. 1 ml. 5 % $CuSO_4$ was added to the contents of the centrifuge tube, which were then made up to 10 ml., the calibration of the tubes used being carefully checked. The contents were mixed by shaking and centrifuged; the clear supernatant fluid was then drawn off for colorimetric estimation.

The serum was separated from a sample of oxalated blood immediately after withdrawal from the vein, and the total protein in the serum was determined by the biuret method as rapidly as possible, and afterwards at frequent intervals until the first rapid change in the serum appeared to be slowing down. The results, expressed as percentages of the true value as shown by Kjeldahl estimations, are given in Table 6. It will be seen

Table 6. *Effect of age of serum on results given by biuret method for total protein*

Results given as percentage of true value as determined by Kjeldahl estimations.

Time after collection of blood	Biuret result by Tintometer [Harrison]	Biuret result by Klett colorimeter [Robinson & Hogden]
1 hr.	51	—
2 „	73	71
3 „	91	—
4 „	90	80
5 „	88	—
24 „	86	81
3 days	86	—

that with the biuret method, as with Greenberg's method, the values gradually increase as the serum ages. Because of the greater experimental error the evidence was less convincing than with the Greenberg method, and we therefore decided to apply spectroscopic methods in the hope of obtaining more conclusive results.

Spectrophotometric determination of the biuret absorption band

Sizer [1937], using a recording photoelectric spectrophotometer, had determined the absorption band of the biuret colour given by casein, and found a maximum at about 520 mμ. Robinson & Hogden [1940], using a Bausch and Lomb universal spectrophotometer, stated that the maximum was at 560 mμ. A number of workers have observed with the naked eye changes in the biuret colour on standing which might be expected to affect the spectrum. We therefore decided to determine the absorption band of biuret colours given by casein and by serum proteins in solutions of different ages. For this purpose we employed a Hilger-Nutting constant deviation wave-length spectrophotometer, which we calibrated for wave-length measurements against the emission spectrum of a helium lamp, and for density measurements against a set of neutral glasses standardized by the National Physical Laboratory. The solution of the biuret colour, carefully freed from all particles, was placed in a 20 mm. cell of Hilger's type D, whilst a precisely similar cell containing the blank (biuret test carried out on distilled water) was placed in the other beam of light.

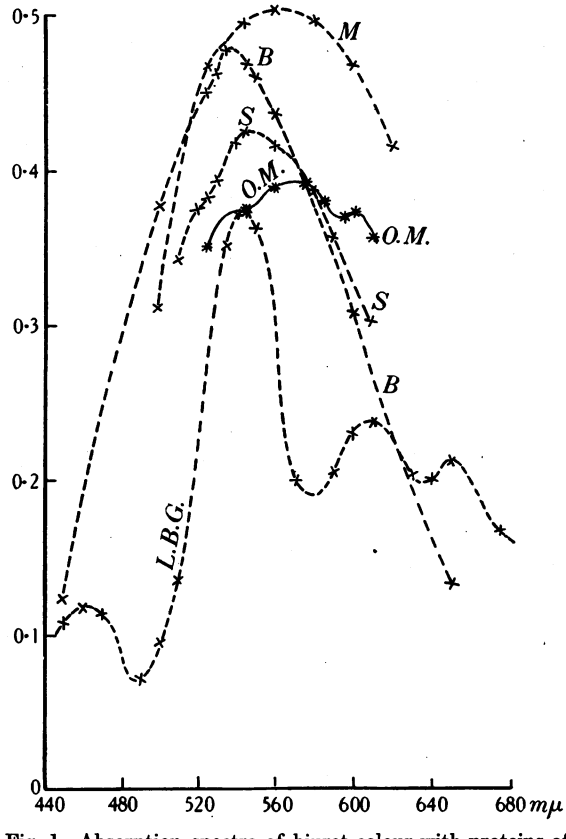

Fig. 1. Absorption spectra of biuret colour with proteins of fresh milk (*M*), of milk after 7 days' storage in refrigerator (*O.M.*), and of fresh serum (*S*), also of biuret itself with $CuSO_4$ and NaOH (*B*) and of 180 Lovibond biuret glass (*L.B.G.*) from the set used for protein estimations. Ordinates represent mean densities; abscissae represent wave-lengths.

Measurements of the density at each wave-length were made independently by two observers, each of whom took 2 pairs of readings with each eye with the biuret colour in the lower beam of light and the blank in the upper beam, followed by another 2 pairs of readings with each eye with the positions of the biuret colour and blank reversed. The first reading in each pair was taken with the lower colour just perceptibly darker than the upper colour, and the second reading with the upper colour just perceptibly darker. This procedure was found to be more satisfactory than attempting to obtain exact matches of colour. The average percentage standard deviation for 4 pairs of readings was brought by careful working below 2, corresponding to a standard deviation of less than 1% for the 16 pairs of readings on which the final figure for the density readings at each wave-length was based. Hence a difference of $2\frac{1}{2}$% (i.e. $2\frac{1}{2}$ times the standard deviation) was considered significant.

Numerous experiments were carried out under different conditions, some of the results being summarized in Fig. 1. The following conclusions were reached.

(1) The absorption maxima for the biuret colour are approximately: on serum proteins 545–555 mμ, on fresh milk proteins 555–565 mμ, and for biuret itself 535 mμ.

(2) As a casein solution ages the absorption maximum of its biuret colour shifts slightly towards longer wave-lengths (e.g. from 560 to 570 mμ after 7 days at 0°).

(3) With solutions of a given protein of the same age the density readings at the maximum are proportional to the concentration of the protein.

The precise maxima for both serum and milk proteins are difficult to determine spectrophotometrically, because of the changes in density and the shift of the peak of the absorption band which are taking place whilst the determination is being made. If a recording photoelectric spectrophotometer had been available, permitting determination to be completed within a short time after separation of the serum, it is possible that the initial maximum would have been found at 520 mμ as Sizer reports. We therefore decided in our quantitative experiments on serum described below to determine the density at 525 mμ, which is probably quite close to the maximum for the biuret colour on freshly separated serum.

Spectrometric study of the effect of ageing of serum proteins on their biuret colour

The spectrophotometer was next employed to study the changes taking place in serum proteins after their removal from the body. The serum was separated from a sample of normal citrated human blood by centrifuging immediately after it had been withdrawn from the vein, and was quickly divided into 3 portions, one of which was placed in the refrigerator, the second in an incubator at 37° and the third was kept at room temperature. The biuret test was at once carried out on a sample of the serum kept at room temperature. The clear supernatant fluid from this test was put in the type D cell and the density determined at 525 mμ by the procedure described above. The first observer started taking readings with the biuret colour in the lower beam, whilst the second observer immediately afterwards started taking readings with the biuret colour in the upper beam. Thus, even if the intensity of the colour were changing during the estimation, the mean of all the readings could be taken as the true value at the time when half the readings had been taken. This was an hour after withdrawal of the blood from the vein, and half an hour after addition of the trichloroacetic acid.

The blood pipettes used to measure the serum had been calibrated with Hg, and corrections were made correspondingly. The calibrations of the centrifuge tubes were also checked.

The estimation was repeated at intervals on the 3 portions of serum, duplicates being run to test the accuracy of the method. The results are recorded in Table 7.

Table 7. *Effect of age of serum and its storage temperature on results given by biuret test using spectrophotometer*

Results given as average densities at 525 mμ.

Age of serum (hr.)	Storage temperature		
	5°	20–25°	37°
1	—	0·641 ±0·008	—
1½	—	0·684 ±0·004	—
2	—	—	0·715 ±0·004
5	0·627 ±0·013	—	0·614 ±0·005
7	0·657 ±0·032	—	—
24	0·672	0·755 ±0·005	—

(1) There was a steady increase in the density readings in the region of maximum absorption, which confirmed the previous findings that the biuret test, like the Greenberg test, gives higher results with older sera.

(2) The rate of this increase depended on the temperature at which the serum was stored.

(3) The mean percentage standard deviation for 7 pairs of duplicate estimations was 2·44, rather higher than was expected from the standard deviation of individual readings.

A difference of 6% was therefore considered significant. The actual increases in density recorded were 11·5% in 2 hr. at 37°, 17·7% in 24 hr. at room temperature, and 7·2% in 24 hr. at 5°. All of these were significant.

(4) The rate of increase in the colour intensity at room temperature from 1 to 1½ hr. was about 7% per hour, much less than had been observed with the tintometer. This was due probably to the much higher experimental error when using the tintometer. The increase observed spectrophotometrically from 1½ to 24 hr. at room temperature was 10·4% as compared with 14% from 2 to 24 hr. in the previous experiment using the Klett colorimeter.

Spectrophotometric study of the effect of ageing of milk proteins on their biuret colour

A sample was taken of the morning milk from Pacified Princess, one of the herd of pedigree Jersey cows on the Ovaltine Dairy Farm. The milk was at once divided into 3 portions, one of which was placed in the refrigerator, the second in the incubator at 37° and the third kept at room temperature. Within 30 min. of the commencement of milking the protein was precipitated with trichloroacetic acid in the usual manner, and the biuret colour was produced and its density at 560 mμ determined in the spectrophotometer. These determinations were repeated at intervals throughout the day on freshly precipitated protein from the 3 samples kept at different temperatures, readings being made in the manner described above, and duplicate precipitations and determinations usually being made. The results, recorded in Table 8, showed the following changes in the intensity of the colour as measured by the density at 560 mμ: (1) a steady increase such as had previously been observed with serum proteins; this was more rapid at higher temperatures; (2) a decrease on storage in the incubator for a prolonged period.

Table 8. *Effect of age of milk and its storage temperature on results given by biuret test using spectrophotometer*

Results given as average densities at 560 mμ.

Age of milk	Storage temperature 5°	15–20°	37°
1 hr.	—	0·540	—
2 ,,	—	—	0·646 ±0·005
3 ,,	—	0·568 ±0·005	—
4½ ,,	0·543 ±0·003	—	—
5½ ,,	—	—	0·816 ±0·019
7½ ,,	0·547	—	—
24 ,,	0·553	0·590 ±0·001	0·661 ±0·034
48 ,,	0·571 ±0·002	—	—
6 days	0·693 ±0·002	—	—

The increase in colour intensity was clearly seen in all 3 portions of the milk, and was in accordance with all our previous observations on serum. The final decrease in the incubator sample had previously been observed with serum kept in the incubator, and probably represented early stages in the decomposition of the protein.

The reliability of the results is shown by the deviations from the mean in 8 pairs of duplicate estimations which are given in Table 8, and by the percentage standard deviations, which ranged from 0·29 to 7·71 and averaged 2·14. The deviations were largest in the incubator samples, possibly due to the greater instability of the protein at higher temperatures, but even here the differences between the 2 and 5½ hr. samples, and between the 5½ and 24 hr. samples, were significant. If the results on the decomposing 24 hr. incubator sample are omitted, the average percentage standard deviation on the remainder of the samples is 1·4, for which a difference of 3–4% would be considered significant. In our opinion this figure gives a fair indication of the accuracy obtainable with the biuret method as we have employed it spectrophotometrically. Fine [1935]

considered that the error of the biuret method using a colorimeter should rarely exceed 5%. He did not, however, take into account any effect due to ageing of the serum. If the tintometer is used in the biuret method as described by Harrison the error may exceed 16%.

In our experiments on the biuret method applied to milk, readings taken at different wave-lengths at different times indicated a slight shift in the peak of the absorption band of the biuret colour as the milk aged, but our results showed that this shift played no significant part in the changes described above.

Spectrophotometer examination of the Lovibond biuret glasses

The deviations we observed in duplicate biuret estimations using Lovibond glasses were much wider than those found by Fine using a colorimeter and by ourselves using either the Klett colorimeter or the spectrophotometer. Suspecting that this might be due to inaccuracies in the Lovibond glasses, we undertook a spectrophotometric examination of the glasses with two objects: (1) to discover whether the colour of the glasses was a fair match for the biuret colour produced by serum or milk proteins, (2) to determine the degree of accuracy with which the various glasses had been graded.

All the determinations were made on a set of glasses, ranging from 20 to 180 on the scale, which was used throughout our experiments, and had also been employed in the College by several hundred students and post graduate workers for determining serum and milk proteins by Harrison's method.

Each glass in turn was carefully cleaned and polished, and placed first in the lower beam, at right angles to it, where two pairs of readings were taken with each eye, by two independent observers, in the manner described above. The glass was then transferred to the upper beam, and the same number of readings taken. Thus the final figure for the density at each wave-length was based on a total of 32 readings, giving results in which a difference of 2–3% was considered significant, the error being greater at certain wave-lengths than at others. The results are plotted in Fig. 1, the curve showing a main peak at about 545 mμ falling within the range of the peaks of serum and milk protein biuret colours, but complicated by the presence of subsidiary peaks at about 460, 605 and 650 mμ which have no counterpart in the true biuret colour. Any error produced by these subsidiary peaks could, however, hardly account for the wide deviations observed in duplicate biuret estimations by the method of Harrison using Lovibond glasses.

Calibration of the Lovibond biuret glasses

Density determinations were made as described above at the peak at 540–545 mμ in each of the glasses graded from 20 to 180. If the glasses had been correctly graded the ratio of the density to the grading of each glass should have been fairly constant. The results

Table 9. *Calibration of Lovibond biuret glasses*

Grading of glasses	Mean density at 540–545 mμ	Grading / Density	Deviation of grading / density from mean for all glasses	% deviation from mean
180	0·374	481	+ 82	20·6
160	0·376	425	+ 26	6·5
140	0·383	365	− 34	8·5
120	0·406	296	−103	25·8
100	0·338	296	−103	25·8
80	0·262	305	− 94	23·6
60	0·189	317	− 82	20·6
40	0·064	625	+226	56·6
20	0·042	481	+ 82	20·6
Mean for all glasses		399	—	23·2
Mean omitting result on 40 and 20 glasses		355	—	16·6

recorded in Table 9 show that the grading of the glasses was unsatisfactory, the deviation from the average density/grading ratio ranging from 6·5 to 56·6%, and averaging 23·2%. In actual practice the 20 and 40 glasses were rarely used, and the average error of 16·6% in the grading of the remainder gives a fairer indication of the error incurred when using this set of glasses. This error is considerably higher than the errors previously observed in the calibration of other Lovibond glasses [cf. Gibson & Harris, 1927; Wokes, 1937], and it must not be assumed to be typical of the general run of Lovibond biuret glasses; it is clear, however, that more calibrations of these glasses are desirable.

Summary

The results given by the biuret and Greenberg methods of estimating protein in serum examined within an hour or two of removal of the blood from the body are lower than the values given by determinations of protein N by Kjeldahl. This applies both to total protein and to albumin or globulin separated from horse or human sera, oxalated or citrated. As the serum ages the results become higher, the rate of increase being more rapid at higher temperatures.

Spectrophotometric determination of the absorption curve of the biuret colour gave the following maxima: fresh serum protein 545–555 mμ, fresh milk protein 555–565 mμ, biuret itself 535 mμ. As casein solutions age the biuret maximum shifts slightly towards longer wave-lengths at about 570 mμ, and indications appear of the presence of two subsidiary peaks at about 540 and 600 mμ.

Density determinations with the spectrophotometer in the region of maximum absorption confirmed the findings obtained with the tintometer and Klett colorimeter that the results increase with ageing of serum, and extended the findings to milk.

Spectrophotometric examination of a set of Lovibond biuret glasses used for protein estimations by Harrison's method showed a main peak at about 545 mμ, also subsidiary peaks at about 460, 605 and 650 mμ which do not appear in the biuret colour on fresh protein and may therefore lead to inaccurate results. A more serious error in this method is inaccurate grading of the glasses, which, judged by the results obtained on the set examined, may lead to errors exceeding 16%.

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